Patient factors influencing the concentration of stromal vascular fraction (SVF) for adipose-derived stromal cell (ASC) therapy in dogs

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Abstract

The objective of this study was to determine whether patient factors influence the concentration of the stromal vascular fraction (SVF) in fat for adipose-derived stromal cell (ASC) therapy in dogs. A total of 1265 dogs underwent adipose collection surgeries by veterinarians for processing by the Vet-Stem laboratory and data on cell counts and patient factors were collected. Body condition score (BCS) and breed size did not significantly affect the viable cells per gram (VCPG) of adipose tissue that represents the viable SVF. Age significantly affected the VCPG, with dogs in age quartile 1 having a significantly higher VCPG than those in quartile 2 ($P = 0.003$) and quartile 4 ($P = 0.002$). Adipose tissue collected at the falciform location had significantly fewer VCPG than tissue collected at the thoracic wall and inguinal locations ($P < 0.001$). When the interaction of gender and location was evaluated, there were significantly fewer VCPG in tissue collected at the falciform location than at the thoracic wall and inguinal locations in female spayed dogs ($P < 0.001$) and male neutered dogs ($P < 0.001$), but not in female intact dogs ($P = 0.743$) or male intact dogs ($P = 0.208$). It was concluded that specific patient factors should be taken into consideration in order to obtain the maximal yield of VCPG from an adipose collection procedure.

Résumé

*L'objectif de la présente étude était de déterminer si des facteurs liés au patient influencent la concentration de la fraction stromale vasculaire (SVF) dans le gras pour le traitement à l'aide de cellules stromales dérivées du tissu adipeux (ASC) chez les chiens. Un total de 1265 chiens ont été soumis à une chirurgie effectuée par des vétérinaires et visant à prélever du tissu adipeux pour traitement par le laboratoire Vet-Stem et des données ont été amassées sur les dénombrements cellulaires et les patients. Le pointage de l'état de chair (BCS) et la taille de la race n'avaient pas d'effets significatifs sur le nombre de cellules viables par gramme (VCPG) de tissu adipeux que représente le SVF viable. L'âge affectait de manière significative le VCPG, les chiens dans le quartile 1 ayant un VCPG significativement plus élevé que ceux dans le quartile 2 (*P *= 0,003) et le quartile 4 (*P *= 0,002). Le tissu adipeux prélevé à la localisation falciforme avait significativement moins de VCPG que le tissu prélevé sur la paroi thoracique ou au niveau inguinal (*P *< 0,001). Lorsque l'interaction du sexe et de la localisation fut évaluée, il y avait significativement moins de VCPG dans le tissu prélevé à la localisation falciforme qu'au niveau de la paroi thoracique et inguinal chez les chiennes stérilisées (*P *< 0,001) et les chiens castrés (*P *< 0,001), mais pas chez les femelles entières (*P *= 0,743) ou les mâles intacts (*P *= 0,208). Il a été conclu que des facteurs spécifiques au patient devraient être pris en considération afin d'obtenir la récolte maximale de VCPG lors d'une procédure de collecte de tissu adipeux.*

(Traduit par Docteur Serge Messier)

Introduction

Mesenchymal stromal cell (MSC) research is a rapidly advancing field in the veterinary and human medical professions. These cells were first identified in bone marrow, but have since been found in a variety of tissues including adipose tissue (1–4). Mesenchymal stromal cells (MSCs) are multipotential cells capable of differentiating into tissue-specific lineages, including chondrogenic, osteogenic, adipogenic, angiogenic, myogenic, and neurogenic lineages (5–9). The versatility of these cells makes them a useful alternative to embryologic stem cells, with less potential for ethical dilemmas.

Adipose tissue has recently gained attention as an abundant source of MSCs. This tissue is a unique reservoir of MSCs as it provides a large quantity of adipose-derived stromal cells (ASCs) in readily accessible locations (10). Studies have shown that the multipotential capacity of ASCs is greater than other tissues of origin such as bone marrow and umbilical cord blood (10–13). It has also been found that there are much higher concentrations of ASCs in adipose tissue than in MSCs in bone marrow (14). Compared to other sources of MSCs, surgical procedures to harvest abundant adipose tissue from ASCs can be done rapidly and with minimal morbidity to the patient. Many of the disease processes that ASCs are used to treat are more common in older patients for whom short anesthetic events and rapid recovery are especially important.

Adipose-derived stromal cells (ASCs) have shown promise in treating osteoarthritis, ligament and tendon injury, fracture nonunion, and spinal cord injuries (6,15–17). Other avenues of research into ASCs include applications in tissue engineering and cloning

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(18). The full potential of ASCs is still unknown as research into ASCs is so novel.

As previously described at the Vet-Stem laboratory, adipose tissue is processed for therapeutic use in stages by mincing the tissue, followed by enzymatic digestion, washing, and centrifugation. After centrifugation, the pellet contains the stromal vascular fraction (SVF) that is composed of a mixture of cells including ASCs, blood cells, pericytes, fibroblasts, and endothelial cells (16). In the dog, ASCs were shown to represent 17 ± 3.2% of the SVF (7).

The ideal site for collecting adipose tissue is one that causes minimal morbidity, consistently has a large amount of fat for extraction, and yields a large SVF proportional to the amount of fat obtained. To our knowledge, there are no studies in the literature evaluating patient factors that influence the collection of SVF from adipose tissue in dogs. The purpose of this study was to evaluate patient factors that may influence concentrations of SVFs in adipose tissue. Veterinary practitioners could use this information to obtain the maximal yield of SVFs from a single adipose tissue harvest procedure. We hypothesized that there would be no difference in SVF yield across the patient factors analyzed. Any statistically significant evidence contrary to the null hypothesis would indicate a patient factor effect on SVF.

Materials and methods

Retrospective study

Data obtained by Vet-Stem (Poway, California, USA) during the processing of all adipose tissue samples from November 2004 to January 2010 were evaluated. Samples of adipose tissue were obtained and processed from 2170 client-owned dogs. The samples were collected by licensed veterinarians in the United States, all of whom were first required to complete a certification course developed by Vet-Stem to ensure that they understood the correct indications for stromal cell therapy as well as proper procedures for collecting, handling, and shipping of samples. The certified veterinarians collected each adipose tissue sample from the location of their choice and using an aseptic technique.

The location chosen for adipose tissue collection was prepared aseptically for surgery. For the inguinal location, a small incision was made in the inguinal region lateral to midline and the subcutaneous fat was dissected out using a scalpel or surgical scissors. The thoracic wall incision was made caudal to the scapula and fat was harvested from the subcutaneous tissue with or without collecting from the fatty deposit underneath the latissimus dorsi muscle as well. Tissue was collected at the falciform using a standard ventral midline incision and collecting the falciform fat after incising the cranial aspect of the linea alba. In all cases, closure was routine and at the veterinarian's discretion. It was recommended that more than 15 g of adipose tissue be collected to improve the chances of obtaining an adequate sample for treatment.

After the adipose tissue was surgically collected, the sample was placed in sterile sample tubes containing 15 mL of phosphate buffered saline (PBS) and shipped by overnight express in a validated, temperature-controlled transport container at 2°C to the Vet-Stem laboratory in California. Samples were shipped on the same day that they were collected. Submitting veterinarians each completed a survey form with information including the patient's breed, age at treatment, site of collection, gender, neuter status, body condition score (BCS) on a 1 to 5 scale, and the location of the adipose tissue collection.

The adipose tissue was processed as previously reported at the Vet-Stem laboratory and the processed samples were then shipped back to the veterinarians for treatment (intralesional and/or intravenous) (16). Information on sample weight, total viable cell yield, viable cell percentage, and viable cells per gram (VCPG) of adipose tissue processed were recorded for each patient after processing.

For the purposes of this study, the VCPG of adipose tissue value represented the viable stromal vascular fraction (SVF) isolated from the sample. Cases in which the samples were submitted without a completely filled out submission form were omitted as were cases in which the BCS was not given as a whole number. There were not enough cases in which the location of adipose tissue collection fell outside the 3 most common locations, i.e., thoracic wall, inguinal, and falciform, for statistical analysis and these cases were therefore not included. Cases in which location of fat collection was not recorded and cases in which samples were collected from multiple locations in the same patient were also omitted from the final analysis. The VCPG was evaluated with regard to gender, neuter status, age, BCS, site of fat collection, and breed size. Age data were available as whole years, without fractional parts, and was therefore treated in quartiles. Since patient weights were not available, the breeds were categorized according to the small, medium, and large sizing scheme of The Kennel Club (London, UK) for the purposes of analysis based on breed size (19). Mixed breed dogs and those breeds not recognized by The Kennel Club were omitted for the purpose of size analysis, but were included in the other statistical analyses.

Statistical analysis

Quantitative descriptive data for metric variables are presented as medians and range and as mean +/− standard deviation (SD) for normally distributed data. Tests of association between categorical variables and metric variables used tests of medians (Mann-Whitney, Kruskall-Wallis). Associations between categorical variables were tested using Pearson's chi-square test of independence or Fisher's exact test. All analyses were done using commercial statistical software (IBM PASW-SPSS v.17, IBM Armonk, New York, USA). For most analyses, a value of $P < 0.05$ was considered significant. Mann-Whitney post-hoc tests after a significant Kruskall-Wallis test used a Bonferroni-corrected alpha as noted in the results section.

Results

Of 2170 dogs for which records were available, 1265 dogs met the inclusion criteria for this study. There were 25 female intact (FI), 577 female spayed (FS), 78 male intact (MI), and 585 male neutered (MN) dogs. Dogs ranged in age from 1 to 18 y with a median age of 8 (mean 7.99 +/− 3.75). There were 99 breeds represented, which included 286 Labrador retrievers, 261 mixed breed dogs, 141 German shepherds, 100 golden retrievers, 45 Rottweilers, 31 mastiffs, 26 border collies, 21 Australian shepherds, 18 Doberman pinschers, 18 Shetland sheepdogs, 14 Siberian huskies, 12 English

Table I. Viable cells per gram (VCPG) of adipose tissue by body condition score (BCS) — total dogs 1265

BCS	Number of dogs	Median	Range	Mean +/− SD
1	25	3.89×10^5	1.61×10^5 to 1.31×10^6	5.12×10^5 +/− 3.14×10^5
2	170	3.69×10^5	4.24×10^4 to 1.38×10^6	4.28×10^5 +/− 2.31×10^5
3	594	3.71×10^5	4.19×10^4 to 3.41×10^6	4.41×10^5 +/− 3.10×10^6
4	385	3.68×10^5	6.00×10^4 to 2.01×10^6	4.27×10^5 +/− 2.37×10^5
5	91	3.47×10^5	1.30×10^5 to 1.12×10^6	3.98×10^5 +/− 1.98×10^5

There was no significant difference in VCPG across categories of body condition score ($P = 0.613$).
SD — standard deviation.

Table II. Viable cells per gram (VCPG) of adipose tissue by collection site — total dogs 1265

Collection site	Number of dogs	Median	Range	Mean +/− SD
Falciform	687	3.38×10^5	4.19×10^4 to 2.09×10^6	3.94×10^5 +/− 2.43×10^5
Thoracic wall	477	4.00×10^5	5.59×10^4 to 1.34×10^6	4.49×10^5 +/− 2.02×10^5
Inguinal	101	4.56×10^5	8.39×10^4 to 3.41×10^5	6.23×10^5 +/− 5.36×10^5

The median VCPG of tissue at the falciform location was significantly lower than tissue at both the thoracic wall and the inguinal locations ($P < 0.001$). There was no significant difference between the median VCPG of tissue at the thoracic wall and the inguinal locations ($P = 0.022$), (Bonferroni adjusted alpha = 0.05/3 = 0.0167).
SD — standard deviation.

Table III. Viable cells per gram (VCPG) of adipose tissue by gender — total dogs 1265

Gender	Number of dogs	Median	Range	Mean +/− SD
Female	602	3.81×10^5	6.00×10^4 to 3.40×10^6	4.42×10^5 +/− 2.85×10^5
Male	663	3.61×10^5	4.19×10^4 to 2.09×10^5	4.26×10^5 +/− 2.59×10^5

There was no significant difference in VCPG of tissue from male and female dogs ($P = 0.170$).
SD — standard deviation.

Table IV. Viable cells per gram (VCPG) by neuter status — total dogs 1265

Neuter status	Number of dogs	Median	Range	Mean +/− SD
Neutered	1162	3.68×10^5	4.19×10^4 to 3.41×10^5	4.28×10^5 +/− 2.68×10^5
Intact	103	3.94×10^5	4.23×10^4 to 2.09×10^6	4.87×10^5 +/− 3.04×10^5

Tissues from neutered dogs had significantly fewer VCPG than tissues from intact dogs ($P = 0.035$).
SD — standard deviation.

bulldogs (12), and 12 boxers. The remaining 280 dogs consisted of groups of less than 10 dogs from various other breeds. Body condition scores (BCSs) recorded ranged from 1 to 5 with a median of 3 (mean 3.27 +/− 0.855). The VCPG of adipose tissue for each body condition score is shown in Table I. There was no significant difference in VCPG across categories of body condition score ($P = 0.613$).

Sample weight was recorded for each submission of adipose tissue. The mean sample weight for all 1265 dogs was 79.39 g +/− 42.66. The mean weight of adipose tissue collected at the falciform location was 91.42 g +/− 48.55, while the mean weights of tissue from the thoracic wall and the inguinal locations were 66.14 g +/− 28.17 and 60.13 g +/− 29.13, respectively.

Adipose tissue collection sites included 687 from the falciform (54%), 477 from the thoracic wall (38%), and 101 from the inguinal location (8%). The VCPG for each location is shown in Table II. The median VCPG of adipose tissue from the falciform location was significantly lower than tissue from both the thoracic wall and inguinal fat harvest sites ($P < 0.001$). There was no significant difference between the median VCPG of tissue from the thoracic wall and the inguinal locations ($P = 0.022$, Bonferroni adjusted alpha = 0.05/3 = 0.0167).

A total of 602 female and 663 male dogs were included in the study and the VCPG for each is shown in Table III. No significant difference in VCPG was found between tissue from male and female dogs ($P = 0.170$).

A total of 1162 neutered dogs and 103 intact dogs was included in the study and VCPG for these dogs is shown in Table IV. Adipose tissue from neutered dogs had significantly fewer VCPG than tissue from intact dogs ($P = 0.035$). When the data were separated into 4 gender categories [male intact (MI), male neutered (MN), female intact (FI), and female spayed (FS)], no significant difference in median VCPG was found between the categories ($P = 0.047$, Bonferroni adjusted alpha = 0.05/6 = 0.0083).

Table V. Viable cells per gram (VCPG) of adipose tissue by age quartile — total dogs 1265

Quartile	Number of dogs	Age range	Median	Range	Mean +/− SD
1	316	1 to 5 y	3.94×10^5	4.19×10^4 to 3.41×10^5	4.81×10^5 +/− 3.44×10^5
2	317	5 to 8 y	3.51×10^5	4.23×10^4 to 1.86×10^6	4.04×10^5 +/− 2.35×10^5
3	316	8 to 11 y	3.75×10^5	5.59×10^4 to 1.61×10^6	4.25×10^5 +/− 2.27×10^5
4	316	11 to 18 y	3.49×10^5	6.77×10^4 to 2.01×10^6	4.11×10^5 +/− 2.52×10^5

Tissue from dogs in age quartile 1 had a significantly higher median VCPG than tissue from dogs in age quartile 2 ($P = 0.003$) and age quartile 4 ($P = 0.002$). SD — standard deviation.

Table VI. Viable cells per gram (VCPG) of adipose tissue by breed size — total dogs 967

Breed size	Number of dogs	Median	Range	Mean +/− SD
Large	810	3.64×10^5	4.19×10^4 to 2.85×10^6	4.31×10^5 +/− 2.68×10^5
Medium	112	4.41×10^5	6.00×10^4 to 1.25×10^5	4.53×10^5 +/− 2.28×10^5
Small	45	4.13×10^5	1.02×10^5 to 1.12×10^6	4.77×10^5 +/− 2.32×10^5

There was no significant difference in VCPG in tissue across breed sizes ($P = 0.058$). SD — standard deviation.

The median age of dogs in the study was 8 y, ranging from 1 to 18 y. Each age quartile and its respective VCPG data are shown in Table V. When cases were age-categorized by quartile, there was a significant difference in median VCPG between at least 2 of the quartiles ($P = 0.003$). Follow-up pairwise Mann-Whitney tests (using an adjusted alpha of $0.05/6 = 0.0083$) were conducted on the median VCPG between each of the age quartiles. Tissue from dogs in age quartile 1 had a significantly higher median VCPG than tissue from dogs in quartile 2 ($P = 0.003$) and dogs in quartile 4 ($P = 0.002$). No other pairwise comparisons between age quartiles showed a significant difference in median VCPG. In particular, the median VCPG between tissue from dogs in quartile 1 and quartile 3 was not significant ($P = 0.153$). Although not conclusive, these results suggest that younger dogs may have higher median VCPG.

For the purposes of breed size analysis, mixed breed and breeds not recognized by The Kennel Club were excluded. A total of 967 dogs met the inclusion criteria 810 (83.8%) of which were classified as large dogs, 112 (11.6%) as medium dogs, and 45 (4.6%) as small dogs. The VCPG for each breed size is shown in Table VI. There was no significant difference in VCPG across breed sizes ($P = 0.058$).

Interactions between location and gender category (MI, MN, FI, and FS) were also evaluated. For tissue collected from the inguinal and the thoracic wall locations, there was no significant difference in the median VCPG across the gender category ($P = 0.916$ and $P = 0.197$), respectively. For tissue taken from the falciform location, however, the median VCPG varied significantly across the gender variables ($P = 0.003$). For tissue from the falciform location, the median VCPG of the neutered males [3.27×10^5, reference range (RR): 4.18×10^4 to 1.44×10^6)] was significantly lower than that of the intact males (4.45×10^5, RR: 4.24×10^4 to 2.09×10^6), ($P = 0.001$). There was no significant difference, however, in median VCPG of tissue from the falciform location in spayed and intact females ($P = 0.189$). Finally, for tissue taken from the falciform, there was no significant difference in median VCPG between males and females, regardless of neuter/spay status (for FI *versus* MI, $P = 0.541$, for FS *versus* MN, $P = 0.308$).

When evaluating the interaction between gender category and location, there was no significant difference in median VCPG of adipose tissue among the 3 locations in the FI group ($P = 0.743$) or in the MI group ($P = 0.208$). For the MN group, there was a significantly lower median VCPG in tissue from the falciform location (3.27×10^5, RR: 4.19×10^4 to 1.44×10^6) than from the thoracic (3.85×10^5, RR: 5.59×10^4 to 1.34×10^6) or inguinal locations (4.37×10^5, RR: 8.39×10^4 to 2.01×10^6) ($P < 0.001$). For the FS group, tissue from the falciform location again had a significantly lower median VCPG (3.36×10^5, RR: 6.00×10^4 to 1.83×10^6) than the thoracic (4.23×10^5, RR: 8.68×10^4 to 1.32×10^5) or inguinal locations (4.71×10^5, RR: 9.69×10^4 to 3.41×10^6) ($P < 0.001$).

Discussion

The results of this study revealed several factors that should be taken into consideration when harvesting adipose tissue for regenerative stem cell procedures. Selecting the surgical site to maximize the VCPG may allow more doses to be obtained from a single adipose tissue collection procedure. This would help to avoid the possibility of there being insufficient cells for the treatment desired. The remaining cells not immediately used for treatment could be banked for future treatments and, with more doses available, the need for costly and time-consuming culturing of the ASCs could be reduced.

Based on the data collected from a large sample of client-owned dogs, BCS did not significantly affect VCPG. A similar result was found in humans, where the frequency of ASCs was not correlated with body mass index (20). For the purposes of this study, BCS was reported by the veterinarians submitting the adipose tissue for processing. While veterinarians are trained to evaluate BCS, the scores they report can vary significantly (21). Because of this inconsistency, significant differences in VCPG among different BCS groups could be missed. Further studies would be required, in which differences in BCS reporting among veterinarians were controlled or a single observer was recording BCS, in order to completely rule out a possible effect of BCS on VCPG.

Breed size was not found to be significantly associated with VCPG. As weight was not listed for the dogs in the sample studied, we used a general breed-sizing scheme to determine if there was a trend in VCPG. There are shortcomings in the sizing scheme,

including variability of sizes within breeds, variability in sizes of the breeds within the categories of the Kennel Club, and exclusion of mixed breed and non-recognized breeds of dogs. Studies on the effect of patient size on VCPG should be done to further determine whether or not there is an association between breed size and VCPG.

Comparisons of VCPG among age quartiles showed significant differences in median VCPG between quartile 1 and quartiles 2 and 4, which suggests that younger dogs may have higher median VCPG. The sample in this study consisted predominately of middle-aged to older dogs as most treatments were directed at chronic musculoskeletal problems, which are more common in older dogs (22). It is possible that a stronger correlation between age and VCPG might be found with larger samples of younger patients. In a human study, no association of age with density of ASCs was found (20). In another study, it was found that the ASCs of younger human patients proliferated at higher rates and were less susceptible to apoptosis, but the function of the ASCs was not evaluated in the present study (23).

Location of adipose tissue collection was significantly associated with VCPG. In the overall sample of dogs, it was found that tissue collected at the falciform location had a significantly lower VCPG than tissue collected at either the inguinal or thoracic wall locations. In a human study, there was no significant difference in VCPG across sites of collection (24). This study does not conflict with our results as only subcutaneous fat samples were obtained in their study so the falciform location was not evaluated. When further evaluated for interactions with gender and neuter status, significant differences were found between tissue from the falciform and tissue from the inguinal and thoracic wall locations in both the male and female neutered groups. There was no significant difference in VCPG between the locations in either the male or female intact groups. The reason for the difference in the intact and neutered groups in the sample was unknown, although it could be speculated that there was a hormonal influence on the VCPG of tissue at the falciform location given the findings. In cats, it has been shown that fat deposition increases in the falciform location after neutering, but the implications for VCPG in tissue taken from the falciform in those cats was not studied (25). Similar studies have not been done in dogs. Further studies would be required to determine the cause of this finding. It is unlikely that human studies would provide insight because few human patients are reproductively altered compared to animals.

In this study, the viable cells per gram (VCPG) of adipose tissue represented the stromal vascular fraction (SVF) of the patients. The percentage of adipose-derived stromal cells (ASCs) was not directly evaluated in the samples. Based on a study of dogs by Neupane et al (7), ASCs represent 17 $\pm$ 3.2% of the SVF. In the subcutaneous, omental, and inguinal locations evaluated, the inguinal ASCs showed significantly less proliferation capabilities (7), although the results should be interpreted with caution as only 3 patients were represented in each group. The ASCs from the omental and subcutaneous locations showed similar differentiation capabilities in vitro (7). In a human study, there were significantly fewer ASCs in the hip/thigh region as a percentage of the SVF than in the subcutaneous fat from the abdomen. There was, however, no difference in differentiation potential of the ASCs between the 2 sites (20). Further studies would be required to evaluate whether ASCs are found at proportionally higher levels in the SVF at different sites. Other factors such as age, breed, or general health status could also affect the concentration or quality of the ASCs in the SVF. This study was not designed to evaluate those factors, however, and future studies would be required to do so.

While obtaining a high number of ASCs is an important consideration when selecting a collection site, the SVF has other cellular components that are also thought to have therapeutic benefits. The adipose-derived SVF also contains endothelial progenitor cells capable of stimulating angiogenesis, monocytes/macrophages that are thought to have anti-inflammatory properties, and T-regulatory cells thought to have an immune-modulatory role (26). Treatment using the complete SVF may therefore have benefits not obtained if treatment is solely with isolated and expanded ASCs.

The main limitation of this study was that it was retrospective in nature. Data in the survey form submitted by veterinarians were sometimes incomplete and therefore cases had to be excluded. As complete medical records from each patient were not available for evaluation, factors such as general health status, comorbidities, and weight at the time of adipose collection could not be analyzed. The large sample size in the study did, however, allow us to thoroughly statistically evaluate the factors that were tested.

When selecting an adipose harvest location for regenerative stem cell therapy, the surgeon must consider patient factors, biologic factors, and his or her own comfort level with the procedure. Patient factors such as previous abdominal surgery could affect the collection procedure. Falciformectomy, which is commonly done during many abdominal surgeries, could greatly reduce the amount of fat present at that location. A patient's fatty deposits can vary significantly. For example, a very thin patient may have minimal subcutaneous fat deposits, which makes the falciform location a more consistent area for sufficient sample availability. The surgeon may also have a different comfort level with surgery in a particular location. Veterinarians are routinely trained to perform ovariohysterectomies and the approach to the falciform location is therefore familiar to most of them. It can be concluded from this study that, in spayed and neutered dogs, the thoracic wall and inguinal adipose collection sites may yield more VCPG than the falciform location. If collecting from the falciform location in an altered patient, more fat should be collected to account for this difference.

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